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## Introduction

Loss-of-function mutations of Whn transcription factor (winged helix nude, alternatively named *Hfh11*, *Foxn1*) result in the nude phenotype (1-3). In addition to a lack of visible hair, abnormally formed epidermis and the absence of a thymus (4), nude mice have defects in the development of mammary glands (5). Female nude mice fail to lactate sufficiently to nourish their pups (6-8). However, little is known about Whn function during mammary gland development or tumorigenesis. We have therefore analyzed the development of the nude mammary gland and determined the expression of Whn during normal mammary gland morphogenesis. Transgenic mice have been created in which Whn is overexpressed in the mammary epithelium to further investigate the role of Whn in the mammary gland development and tumorigenesis.

## Body

### Task 1. Determine the temporal and spatial expression of Whn during mammary gland development

#### Nude mammary glands display defects in development

The development of mammary glands involves branching of the ducts and the formation of lobulo-alveoli (9-11). The ductal tree undergoes rapid growth at the onset of puberty. Highly proliferative structures called terminal end buds (TEB) appear at the end of the ducts, and the ducts lengthen and branch until they form a ductal network extending to the limits of the fat pad. During pregnancy additional ductal branching occurs and lobulo-alveolar structures form by extensive proliferation and differentiation. By late pregnancy, the alveoli fill the majority of the fat pad and begin producing milk.

We have investigated the structure of nude mouse mammary glands during development. Whole mounts were prepared from nude mice and their wild-type littermates at 4, 7 and 10 weeks of age, and during pregnancy. Carmine alum staining was used to identify epithelial structures. The ductal branching is delayed and retarded in the nude mammary glands compared to the wild-type glands at the same age, suggesting that Whn promotes proliferation of the mammary epithelium. In the mammary glands from 4-week old nude mice, the ductal branching has barely initiated and TEBs are infrequently found, while the front of the ductal trees has passed the lymph node in the wild-type glands (Figure 1A and 1D). 7-week nude mammary glands also contain fewer TEBs than the wild-type glands (Figure 1B and 1E). By 10 weeks of age, the epithelial ducts have reached the margins of the fat pad in the wild-type mammary glands as well as in the nude glands. However, the degree of ductal branching is significantly reduced in nude mice (Figure 1C and 1F). Wild-type mammary glands undergo further branching during pregnancy, and increase greatly in size because of the extensive proliferation of alveolar buds. Unlike wild-type mice, pregnant nude glands contain fewer branches, which are densely packed (Figure 2A and 2C). In addition the nude glands are significantly smaller, approximately half the size of the wild-type.



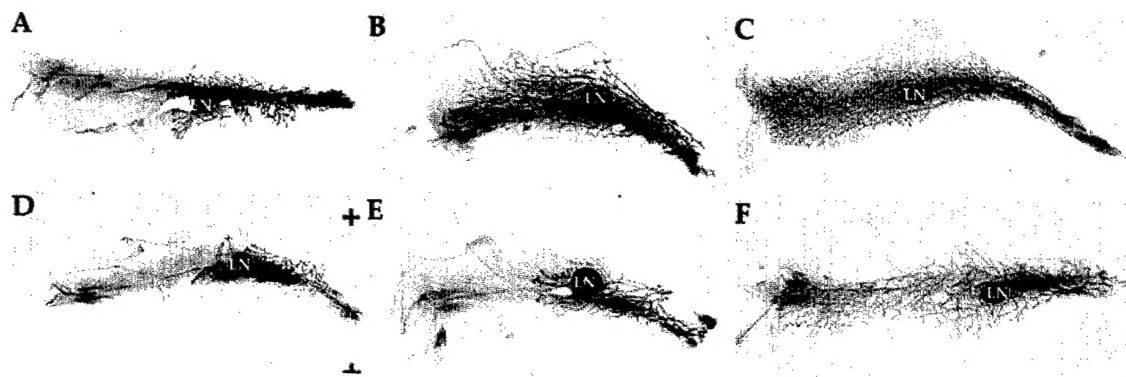


Figure 1. Whole mounts of the number 4 inguinal gland were prepared from wild-type (A-C) and nude (D-F) virgin mice at 4 weeks (A, D), 7 weeks (B, E) and 10 weeks (C, F) of age. Whole mounts are stained with carmine alum, and oriented with the nipple towards the right, and LN indicates the position of the lymph node.

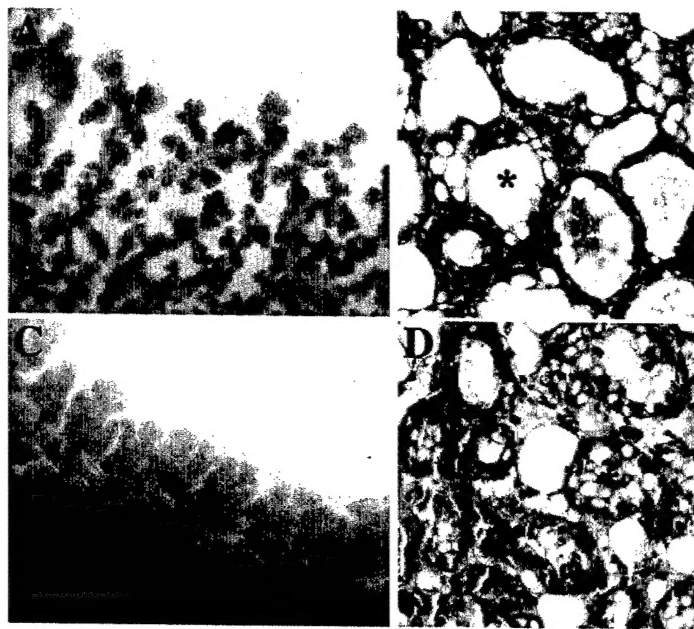


Figure 2. Mammary tissue was taken from nude and wild-type mothers a few hours after parturition. Whole mounts (A and C) were stained with carmine alum and paraffin sections (B and D) were stained with hematoxylin and eosin. Nude mammary glands (C) contain fewer branches and branches are densely packed compared to the wild-type glands (A). Wild-type glands (B) possess large alveoli and the empty lumens (\*) indicate that milk is removed by suckling pups. Nude mammary tissue at the same magnification (D) illustrates the reduced size and abnormal appearance of the lobulo-alveoli. The lumens (\*) are still full of milk.

Mammary epithelial cells differentiate into milk-secreting cells during late pregnancy. In wild-type glands, numerous lobulo-alveoli are formed and milk is secreted into the lumen of the alveoli. Following the birth of the pups, milk is emptied from the lumen by the suckling pups. Sections from nude mammary glands show that lobulo-alveoli are smaller than those in wild-type mice and lumens remain full of secretions despite the presence of pups attempting to suckle (Figure 2B and 2D). This result suggests that *Whn* also affects the differentiation of mammary epithelial cells.

### Spatial and temporal expression of *Whn* in the mammary epithelium

We performed RT-PCR to determine the temporal expression pattern of *Whn* during mammary gland development. RNA was extracted from wild-type mammary glands at various stages of development, and first-strand cDNA was synthesized. The presence of *Whn* transcript was determined by PCR with primers corresponding to nucleotides 774-796 and 1449-1470 of the *Whn* cDNA. RT-PCR with primers specific to  $\beta$ -actin, a constitutively expressed gene, was performed to ensure the integrity of RNA. *Whn* transcripts can be detected as early as 3 weeks (Figure 3). At this age the mice are entering puberty, the terminal end buds are forming and the proliferation rate of the epithelium is increased. *Whn* expression persists throughout the time that the ductal network is developing, and is no longer seen once the ducts have reached the limits of the fat pad and development is complete (after 8 weeks, Figure 3). During pregnancy *Whn* expression starts from day 15, and persists through the birth of the pups and the onset of lactation (Figure 3). Morphological studies have shown that late pregnancy stage is the period when mammary tissue is actively growing and differentiating. Taken together, these results suggest that *Whn* is involved in both proliferation and differentiation of the mammary epithelium. This is consistent with the previous work from our laboratory showing that *Whn* has roles in both proliferation and differentiation of keratinocytes in the skin (12-14).

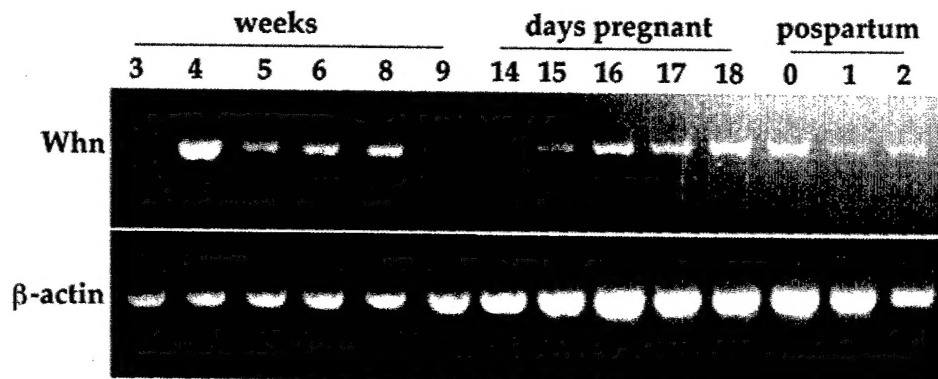


Figure 3. *Whn* is expressed in the mammary gland during ductal development and pregnancy. RNA was purified from mammary glands taken from virgin or pregnant mice at the indicated times, and *Whn* transcript detected by RT-PCR. RT-PCR for  $\beta$ -actin transcript is used as a positive control.

## Abnormalities in nude mammary glands are not due to changes in hormonal levels

The development of the mammary glands is highly regulated by the coordinate action of a number of hormones, such as estrogen, progesterone, prolactin, oxytocin, activins/inhibins and other members of the TGF- $\beta$  family (9, 10, 15). Studies with ovariectomized mice and gene knockout mice have shown that estrogen is required for ductal outgrowth (16, 17) and that progesterone is important for both the ductal growth and the lobulo-alveolar differentiation (18, 19). To investigate whether the defects of the nude mammary glands is caused by changes in systemic hormonal level, we measured the serum levels of estradiol and progesterone during virgin development and pregnancy. Table 1 shows that there is no significant difference in the serum level of these two hormones between the wild-type and nude mice at any tested time point, thus the defects in nude mammary gland development are due to an intrinsic deficiency in the gland.

**Table 1 Hormone levels in nude and wild-type mice<sup>a</sup>**

Hormone tested	Age of the mice <sup>b</sup>	wild-type	Nude	<i>p</i> value <sup>c</sup>
Estradiol (pg/mL)	virgin	95.6 $\pm$ 36.7 <sup>d</sup> n = 8	100.5 $\pm$ 35.8 n = 10	0.78
	pregnant	121 $\pm$ 26.3 n = 5	120 $\pm$ 24.7 n = 5	0.95
Progesterone (ng/mL)	virgin	12.2 $\pm$ 12.5 n = 10	5.35 $\pm$ 7.44 n = 12	0.12
	pregnant	64.2 $\pm$ 33.3 n = 5	23.3 $\pm$ 21.5 n = 5	0.05

a, Serum samples were collected from nude mice and their heterozygous littermates at indicated time points and tested for the two hormones. Heterozygous are used as wild-type control since they are phenotypically normal.

b, Virgin mice are between the age of 4 to 8 weeks, which is the time that mammary glands are undergoing robust branching. Pregnant mice are between 14.5 to 18.5 days of pregnancy, when numerous lobulo-alveoli are forming.

c, Single factor anova analysis was done for the data from each time point between nude and wild-type mice with Microsoft Excel. Significant difference is determined to be existing between the two groups when  $p < 0.05$ . No significant difference is seen at the given time points for the two hormones tested.

d, Average  $\pm$  standard error of mean, n = number of samples collected

## Whn is not required for the transcription of $\beta$ -casein, $\alpha$ -lactalbumin, and whey acidic protein

Terminal differentiation of the alveolar epithelial cells converts them into milk secreting cells. Since Whn is a transcription factor that affects the differentiation of epithelial cells, we investigated whether Whn affects transcript levels of milk proteins  $\beta$ -casein,  $\alpha$ -lactalbumin and Whey Acidic Protein (WAP) (20-22) by Northern blot analysis. Total RNA was prepared from pregnant and postpartum wild-type and nude mammary glands, and the expression level of each protein were examined. Although each transcript displayed unique temporal expression pattern, the levels were similar in both wild-type and nude mammary tissue (Figure 4). The decreased transcripts in the nude mice at 2 day postpartum is likely to be caused by involution in the nude mammary glands. Thus Whn is not essential for the transcription of these milk proteins.

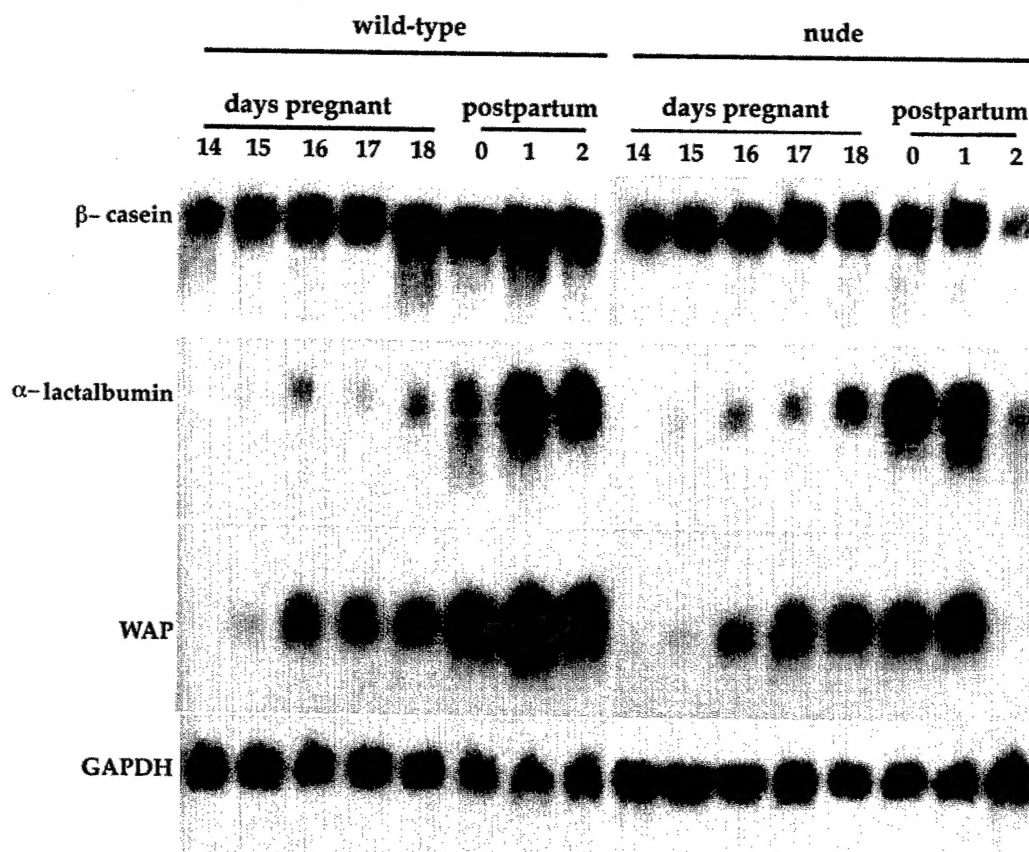


Figure 4. Whn is not required for the transcription of milk proteins. RNA was purified from mammary glands taken from wild-type or nude mice at the indicated times and northern blots were hybridized with probes for the indicated transcripts. No difference in the level of milk protein transcripts were observed between nude and wild-type, except at 2 day postpartum when levels of all three milk protein transcripts decreased in the nude mouse, probably due to the onset of involution.

## Defects in nude mammary glands can be rescued by *Inv-Whn* transgene

Transgenic mice have been generated in our laboratory to investigate *Whn* function in skin (12). These mice carry a transgene in which *Whn* is expressed from the involucrin (*Inv*) promoter. Involucrin is a component of the cornified envelope present in the epidermis, hair follicles, urothelium and other stratified epithelia (23-25). *Inv-Whn* transgenic animals were crossed to nude mice to generate nude double mutant mice. Female mice carrying both mutations are capable of nursing their pups, indicating that the transgene can compensate the loss of endogenous *Whn* in the mammary glands. RT-PCR was used to identify the expression of the transgene and endogenous involucrin gene in the mammary glands. The *Whn* transgene was detected in the mammary tissue from all double mutant mice, in both lactating and mature non-pregnant stages (Figure 5). In addition, transcripts of endogenous involucrin gene were detectable in the mammary glands from all mice, including mature non-pregnant wild-type and nude mice. Thus involucrin is normally expressed in the mammary glands. This rescue demonstrates that the abnormalities in the nude mammary glands are due to an intrinsic defect in the mammary epithelium.

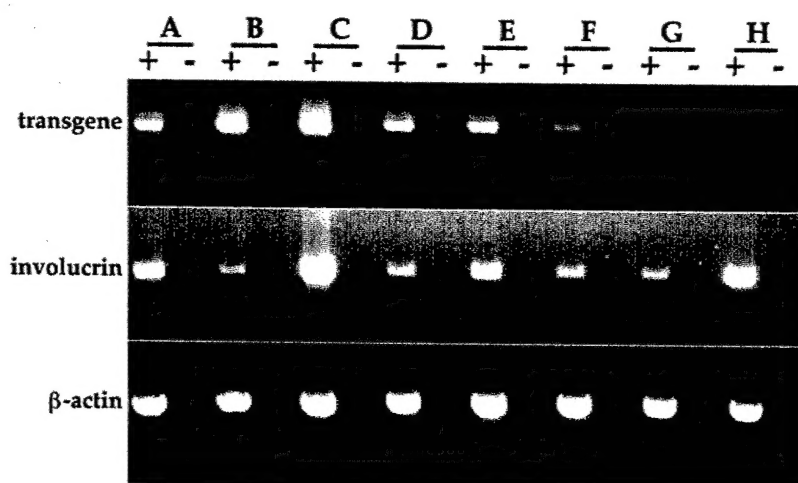


Figure 5. Involucrin and the *Inv-Whn* transgene are expressed in the mammary gland. RNA was isolated from mature non-pregnant nude mice carrying the *Inv-Whn* transgene (A-E), a lactating mouse expressing the *Inv-Whn* transgene (F), a mature non-pregnant wild-type (G) and a mature non-pregnant nude (H). RT-PCR indicated the expression of the *Inv-Whn* transgene in the mammary tissue of all mice positive for the transgene. The involucrin transcript was detected in the mammary tissue of all mice regardless of the presence of the transgene. Lanes with "+" are experiments with reverse transcriptase, and lanes with "-" are negative controls with no reverse transcriptase. The  $\beta$ -actin transcript was used as a positive control.

## Task 2. Create and analyze transgenic mice that overexpress Whn in mammary epithelia

To further investigate the role of Whn in the mammary glands, we have created mice that overexpress Whn in the mammary epithelium. The construct for making these transgenic mice contains the complete mouse *Whn* cDNA under the control of the mouse mammary tumor virus (MMTV) promoter. MMTV promoter includes all the necessary cis-elements to target expression to the mammary epithelia (26, 27). A Flag epitope tag was placed at the translation start site so we can distinguish the ectopically expressed Whn from the endogenous protein. Transgenic mice were produced by pronuclear injection of the MMTV-*Whn* construct into C57BL6 x DBA fertilized oocytes.

18 founder mice were identified by PCR using primers to the *Whn* cDNA, and Southern blotting proved that the transgene they carry is intact. 12 of these mice transmitted the transgene to the next generation. Mammary gland extracts were made from transgenic mice and non-transgenic controls, and were examined for the expression of Whn by immunoprecipitation using antibodies to the Flag tag, followed by Western blotting with antibodies to Whn. Figure 6 shows that Whn was overexpressed in the transgenic mammary glands.

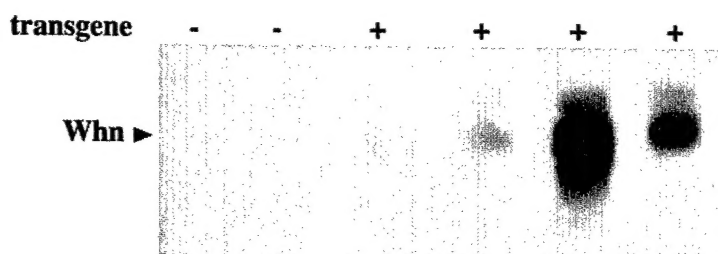


Figure 6. Mammary tissue lysates were prepared from transgenic mice and wild-type littermates. Lysates were incubated with antibodies to the Flag epitope, and immune complexes recovered with protein-G sepharose, separated by SDS-PAGE and transferred to PVDF membranes. Membranes were incubated with antibodies to Whn, and proteins were visualized using HRP-conjugated secondary antibodies and the enhanced ECL detection system. Whn protein was detected as multiple bands because of posttranslational modification (14 (Appendix 1)).

Four lines of MMTV-*Whn* transgenic mice with the highest levels of transgene expression were chosen for further analysis. To investigate the effects of Whn overexpression on mammary gland development whole mounts were prepared from transgenic mice and wild-type littermates at various ages. After extensive analysis, no differences in morphology were observed between the transgenic mice and their wild-type littermates. Overexpression of Whn has not affected the normal function of the mammary glands, as transgenic females are capable of nursing their pups. No abnormalities of the mammary gland were found in the transgenic mice even when we



increased the levels of overexpression by mating siblings to generate mice homozygous for the transgene.

These transgenic mice were also observed for mammary gland tumorigenesis. No spontaneous mammary tumors were seen in these mice during the 2-year observation. Thus, overexpression of Whn is not sufficient for mammary tumor formation.

### Task 3. Analysis of Whn protein in human breast cancer sections and cell lines.

Human Whn is 85% identical to its murine homolog (2), and in collaboration with others, we showed that a nonsense mutation in human *Whn* is associated with congenital alopecia and severe T cell deficiency (28). Thus, the loss of Whn activity in humans leads to a disease that closely resembles the nude phenotype, demonstrating the conservation of Whn function. The human gene maps to chromosome 17q (2, 29), a region frequently amplified in human breast cancer (30-34), and we have shown that overexpression of Whn in the epidermis leads to a hyperproliferative phenotype in mice (12). Therefore, we have investigated the expression of *Whn* in human breast cancer cell lines. Two cell lines derived from normal human mammary epithelium and several cell lines derived from breast tumors were obtained from the American Type Culture Collection. Cell lysates were prepared and subjected to immunoprecipitation with Whn specific antibodies. Levels of Whn were determined by immunoblotting the precipitated proteins. Figure 7 shows that Whn was detected at low levels in the normal breast cell lines, Hs

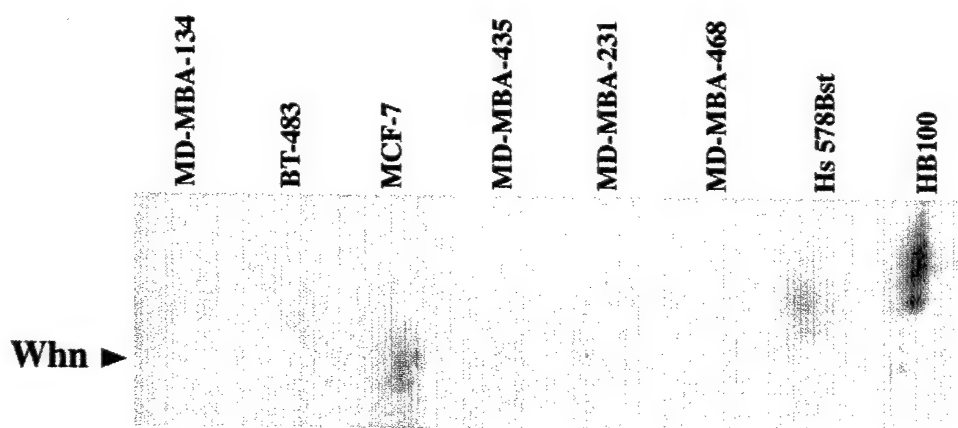


Figure 7. Whn is detected in cell lines derived from normal human breast and breast cancers. Lysates from cultured cells were incubated with antibodies to Whn, and immune complexes recovered with protein-G sepharose, separated by SDS-PAGE and transferred to PVDF membranes. Membranes were incubated with the same antibodies, and proteins were visualized using HRP-conjugated secondary antibodies and the enhanced ECL detection system. Hs 578Bst and HB100 are normal cell lines and the others are cancer cell lines.

578Bst and HB100, and in several of the tumor cell lines, e.g., MCF-7 and MDA-MB 435. Tumor cell lines MD-MBA 134 and BT-483 nearly have no detectable Whn expression. These data show that Whn expression is highest in the normal breast cell lines, which suggests that the loss or decrease of Whn expression may be involved in malignant progression. Although overexpression of Whn resulted in increased proliferation in the epidermis (12), our data from MMTV-*Whn* transgenic mice indicated that increased expression of Whn is not related to tumor formation. On the other hand, Whn promotes the differentiation of the cell in which it is expressed (13, 14 (Appendix 3)). Thus the loss of Whn function may result in a cell that has increased proliferative potential and can contribute to the formation of poorly differentiated tumors.

### **Key research accomplishments**

- Determined temporal expression of Whn during early development and pregnancy
- Illustrated that the abnormalities of nude mammary glands are due to an intrinsic epithelial defect
- Discovered that overexpression of Whn in mammary glands does not induce tumor formation
- Identified the expression of Whn in human breast cancer cell lines
- Rescue of nude mouse lactation defects by expression of Whn transgene in mammary glands

### **Reportable outcomes**

- Poster presentation at Massachusetts Department of Public Health Breast Cancer Research Symposium, April 2000
- Poster presentation at Cutaneous Biology Research Center Retreat, April 2001
- Poster presentation at Era of Hope Department of Defense Breast Cancer Research Program Meeting, September 2002
- Generation of transgenic mice expressing Whn from the MMTV promoter
- Generation of transgenic mice expressing Whn from the involucrin promoter



## Conclusions

We have shown here that the *Whn* transcription factor is critical for the development of mammary glands. Loss of this protein in the nude mammary glands leads to retarded ductal branching and failure to lactate. Expression of *Whn* is detectable at times when the glands undergo robust ductal outgrowth and lobulo-alveolar development, suggesting *Whn* is involved in both proliferation and differentiation of the mammary epithelial cells. Systemic hormone levels were comparable between nude and wild-type mice, and the transcription of milk proteins, which are under hormonal control, was similarly unaffected in nude mice. Thus the lactation defects in the nude mice are due to an intrinsic deficiency in the mammary glands. Furthermore, these defects can be rescued by the expression of a *Whn* transgene in the mammary epithelial cells, indicating that *Whn* is required for the normal development and function of mammary epithelia.

We have created MMTV-*Whn* transgenic mice that overexpress *Whn* in the mammary epithelium to study the role of *Whn* in tumorigenesis. No transgenic mice were found to develop mammary tumors, thus overexpression of *Whn* does not correlate with tumor formation. Consistent with this result, we have found that *Whn* expression in several human breast cancer cell lines is lower than in normal breast cell lines. These data indicate that loss of *Whn* expression may contribute to tumorigenesis of mammary epithelial cells.

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## Appendix 1

### Publications and Meeting Abstracts

Baxter R. M., and Brissette J. L. (2002). Role of the *nude* gene in epithelial terminal differentiation. *J. Invest. Dermatol.* 118:303-309

Baxter R. M., and Brissette J. L. (2000). Mutations in the *Whn (nude)* gene result in impaired mammary gland development and lactation. Massachusetts Department of Public Health Breast Cancer Research Symposium. (Abstract)

Baxter R. M., and Brissette J. L. (2001). Mutations in the *Whn (nude)* gene result in impaired mammary gland development and lactation. Cutaneous Biology Research Center Retreat. (Abstract)

Han R., Baxter R. M., and Brissette J. L. (2002). *Whn* gene mutation results in impaired mammary gland development. Era of Hope Department of Defense Breast Cancer Research Program Meeting. (Abstract)

## Appendix 2

Personnel receiving pay:

Ruth Baxter, Ph.D.

Rong Han, Ph.D.

# Role of the *Nude* Gene in Epithelial Terminal Differentiation

Ruth M. Baxter and Janice L. Brissette\*

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**Loss-of-function mutations in *Whn* (*Hfh11*, *Foxn1*), a winged-helix/forkhead transcription factor, cause the nude phenotype, which is characterized by the abnormal morphogenesis of the epidermis, hair follicles, and thymus. To delineate the biochemical pathway of *Whn*, we investigated its upstream regulation and downstream effects using primary keratinocytes from wild-type and transgenic mice. The transgenic animals express *whn* from the involucrin promoter, which is active in keratinocytes undergoing terminal differentiation. In wild-type cultures, as in the epidermis, *Whn* was induced during the early stages of terminal differentiation and declined during later stages. In transgenic keratinocytes, *whn* overexpression altered the terminal differentiation program, stimulating an early differentiation marker**

**(keratin 1) and suppressing later markers (profilaggrin, loricrin, and involucrin). These results suggest a role for *Whn* in the stepwise or temporal regulation of differentiation, as *Whn* can ensure that the differentiation program is carried out in proper sequence. Before the start of differentiation, *Whn* levels were suppressed by the p42/p44 mitogen-activated protein kinase cascade, and this signaling pathway was rapidly inactivated as differentiation began. Thus, as keratinocytes commit to terminal differentiation, mitogen-activated protein kinase signaling decreases, which permits the induction of *Whn*; *Whn* then activates early features of the differentiation program. Key words: *Foxn1/Hfh11/keratinocyte/mitogen-activated protein kinase/Whn*. *J Invest Dermatol* 118:303–309, 2002**

The epidermis and hair follicles are related epithelial structures with many similarities in their development, organization, and self-renewal (reviewed in Fuchs, 1990; Hardy, 1992). Both originate from proliferative cell populations that exhibit relatively few differentiated features and generally border the dermis. These proliferative cells act as the progenitors of postmitotic cell types, which undergo terminal differentiation and form each structure's functional components (e.g., the stratum corneum and hair). Though distinct from each other, the epidermal and follicular differentiation programs lead to comparable cellular changes, such as the acquisition of a flattened shape, the accumulation of keratins, and the breakdown of the nucleus. To produce a differentiated cell, these programs proceed through an ordered series of events, implying stepwise or temporal regulation of the process. While many factors are known to influence the formation of cutaneous epithelia, much remains unknown about the commitment of a progenitor cell to terminal differentiation, and the subsequent stepwise control of the differentiation program.

Nude mice and rats are characterized by the abnormal development of the epidermis, the lack of visible hair, and the absence of a thymus (Flanagan, 1966; Pantelouris, 1973; Köpf-Maier *et al*, 1990). This phenotype results from inactivating mutations in a single gene, originally designated *whn* (winged-helix nude) or *hfh11* (hepatocyte nuclear factor 3/forkhead homolog 11) (Nehls *et al*, 1994, 1996; Segre *et al*, 1995), and recently renamed *foxn1*

(forkhead box n1) (Kaestner *et al*, 2000). The human and murine *Whn* proteins are 85% identical (Schorpp *et al*, 1997), and a nonsense mutation in human *whn* is associated with congenital alopecia and severe T cell deficiency (Frank *et al*, 1999). Thus, loss of *Whn* activity in humans results in a disease that closely resembles the nude phenotype, demonstrating the conservation of *Whn* function.

*Whn* is a member of the winged-helix or forkhead family of transcription factors, which share a highly conserved stretch of 100 amino acids (Kaufmann and Knochel, 1996). This conserved region forms a modified helix–turn–helix domain that mediates DNA binding (Kaufmann and Knochel, 1996). The negatively charged C-terminal domain of *Whn* can stimulate transcription when fused to the Gal4 DNA binding domain (Brissette *et al*, 1996; Schüddekopf *et al*, 1996; Schlake *et al*, 1997), indicating that *Whn* functions as a transcriptional activator.

In murine epidermis and hair follicles, *nude* mutations impair terminal differentiation, as several differentiated structures (such as the stratum corneum, inner root sheath, hair cortex, and hair cuticle) fail to form properly (Köpf-Maier *et al*, 1990). During skin development, epithelial cells induce *whn* expression as the first signs of terminal differentiation appear (Lee *et al*, 1999). In mature skin, *whn* expression is maintained at sites displaying the early stages of terminal differentiation, such as the first suprabasal layer of the epidermis and the supramatrical region of the hair bulb. Though mainly restricted to postmitotic cells, *whn* expression is also detected within progenitor cell compartments, marking a subset of epithelial cells in the basal epidermal layer, outer root sheath, and hair matrix. Based on the distribution of Ki-67, a nuclear marker of proliferation, a small number of multiplying cells express *whn* (Lee *et al*, 1999). Thus, *whn* expression seems to encompass the transition from proliferation to differentiation, and *whn* activation may be one of the first steps in the terminal differentiation pathway. Consistent

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Abbreviations: MAPK, mitogen-activated protein kinase.

with its epithelial specificity in the skin, *whn* expression is also detected in the epithelial cells of several other organs, such as the thymus, nail, tongue, palate, nasal cavity, and teeth (Lee *et al*, 1999). In addition, *whn* homologs were identified in species that do not possess hair follicles or a thymus, such as amphioxus (Schlake *et al*, 1997) and fruit flies (Strodick *et al*, 2000; Sugimura *et al*, 2000). Given *whn*'s phylogenetic distribution as well as its murine expression pattern, *whn* may control a common or fundamental property of epithelia (Lee *et al*, 1999).

In previous studies, transgenic mice were generated in which *whn* was placed under the control of the involucrin (*inv*) promoter (Prowse *et al*, 1999). Involucrin, a component of the cornified envelope (Rice and Green, 1979), is present in many stratified epithelia and serves as a marker of terminal differentiation in the epidermis and hair follicles (Rice and Green, 1979; Walts *et al*, 1985; de Viragh *et al*, 1994). Consistent with the *inv* expression pattern, the *inv-whn* mice develop severe, often lethal, abnormalities in the skin and urinary tract. These abnormalities include flaky, shiny skin, misshapen, truncated hair shafts, and a loss of epidermal barrier function, all of which indicate defects in differentiation. Thus, *whn* overexpression, like the loss of *whn* function, disrupts the terminal differentiation of the epidermis and hair follicles.

In this study, we examined Whn's role in differentiation using murine keratinocyte cultures. Similar to its cutaneous expression pattern, endogenous Whn is induced as primary keratinocytes initiate terminal differentiation. In *inv-whn* cultures, *whn* overexpression stimulates an early differentiation marker and suppresses later markers, implicating Whn in the stepwise or temporal control of the differentiation program. Furthermore, the mitogen-activated protein kinase (MAPK) cascade functions as an upstream regulator of Whn, as this pathway suppresses Whn levels in proliferating keratinocytes and becomes inactivated with the onset of differentiation. Thus, this study identifies an early sequence of events in the conversion of progenitor keratinocytes to a differentiated phenotype, as the inactivation of MAPK leads to the induction of Whn, which in turn stimulates initial features of differentiation.

## MATERIALS AND METHODS

**Cells** For analyses of *whn* expression and function, primary keratinocytes were prepared from newborn *inv-whn* mice and their wild-type littermates. Pups were genotyped by polymerase chain reaction of tail DNA with primers corresponding to nucleotides 774–796 and 1449–1470 of the mouse *whn* cDNA (Nehls *et al*, 1994). Keratinocytes were grown in minimal essential medium containing a low calcium concentration (0.05 mM), 4% chelex-treated fetal bovine serum, and 10 ng epidermal growth factor per ml as described previously (Hennings *et al*, 1980). Terminal differentiation was induced by the addition of  $\text{CaCl}_2$  (final concentration 2 mM). To block MAPK signaling, cells were treated with the MEK inhibitors U0126 (Promega, Madison, WI; final concentration 1  $\mu\text{M}$ ) or PD098059 (LC Laboratories, Woburn, MA; final concentration 50  $\mu\text{M}$ ).

**Immunoprecipitations, immunoblotting, and antibodies** For analysis of Whn protein, cells were lysed in buffer containing 50 mM Tris, pH 8.0, 150 mM NaCl, 1% nonidet P40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 2 mM phenylmethylsulfonyl fluoride, and 1 mM  $\text{Na}_3\text{VO}_4$  (lysis buffer). Immune complexes were recovered with protein G-sepharose. Sepharose beads were washed twice in lysis buffer, once in buffer containing 100 mM Tris, pH 7.4, 500 mM NaCl, and twice in buffer containing 10 mM 1,4-piperazine-diethanesulfonic acid (PIPES), pH 7.0, 100 mM NaCl.

For analysis of differentiation markers, cells were lysed in buffer containing 100 mM Tris, pH 6.8, 4% SDS, 20% glycerol, and 0.2% bromophenol blue. Protein levels were normalized using Bradford assays (Bio-Rad Laboratories, Hercules, CA). Proteins were electrophoresed on SDS-polyacrylamide gels and transferred to Immobilon membranes (Millipore, Bedford, MA). Following incubation with primary antibodies, proteins were visualized using horseradish peroxidase-conjugated secondary antibodies (1:2000; Amersham Pharmacia Biotech, Piscataway, NJ) and the enhanced chemiluminescence (ECL) detection system (NEN, Boston, MA).

For analysis of MAPK phosphorylation, cells were lysed in buffer containing 50 mM Tris, pH 7.5, 1 mM ethylenediamine tetraacetic acid, 1 mM ethyleneglycol-bis-( $\beta$ -aminoethylether)- $N,N,N',N'$ -tetraacetic acid, 0.5 mM  $\text{Na}_3\text{VO}_4$ , 0.1%  $\beta$ -mercaptoethanol, 1% Triton X-100, 50 mM NaF, and 2 mM phenylmethylsulfonyl fluoride (MAPK assay buffer A). Protein levels were normalized and immunoblots performed as described above.

Polyclonal antibodies were raised against the full-length murine Whn protein fused to glutathione S-transferase. To generate the fusion protein, the *whn* cDNA (nucleotides 97–2038) was subcloned into the pGex2T vector (Promega), and the construct was expressed in bacteria. The Whn antibodies were affinity purified at Strategic Biosolutions Inc. (Ramona, CA) and used at 1:500 dilution for immunoblots. Antibodies against keratin 1 (K1; 1:1000), keratin 5 (K5; 1:1000), loricrin (1:1000), and filaggrin (1:1000) were kindly provided by Dr G.P. Dotto (Massachusetts General Hospital, Charlestown, MA). Involucrin antibodies (1:1000) were from Covance (Richmond, CA), antibodies to phosphotyrosine (0.1  $\mu\text{g}$  per ml), phosphoserine (0.5  $\mu\text{g}$  per ml), and phosphothreonine (0.5  $\mu\text{g}$  per ml) were from Zymed (South San Francisco, CA), and monoclonal antibodies against Flag (M2; 10  $\mu\text{g}$  per ml) were from Sigma (St Louis, MO). Polyclonal antibodies that recognize total p42/p44 MAPK (phosphorylation-independent antibodies) were used at 1:500 for immunoblots and 4  $\mu\text{g}$  per immunoprecipitation (Upstate Biotechnology, Lake Placid, NY). Antibodies specific for the phosphorylated forms of the p42/p44 MAPK (0.1  $\mu\text{g}$  per ml) were from Cell Signaling Technology (Beverly, MA).

**MAP kinase assay** For analysis of MAP kinase activity, cells were lysed in MAPK assay buffer A. Protein levels were normalized using a Bradford assay (Bio-Rad Laboratories), and MAPK was immunoprecipitated from 1 mg total protein using 4  $\mu\text{g}$  antibody to total p42/p44 MAPK (Upstate Biotechnology). Immune complexes were recovered with protein G sepharose beads, washed twice in MAPK Assay Buffer A, and washed once with Assay Dilution Buffer [ADB; 20 mM 3-[N-morpholino]propanesulfonic acid (MOPS), pH 7.2, 25 mM  $\beta$ -glycerophosphate, 5 mM ethyleneglycol-bis-( $\beta$ -aminoethylether)- $N,N,N',N'$ -tetraacetic acid, 1 mM  $\text{Na}_3\text{VO}_4$ , and 1 mM dithiothreitol]. Assays were carried out in 50  $\mu\text{l}$  ADB containing 4  $\mu\text{M}$  protein kinase C inhibitor peptide, 0.4  $\mu\text{M}$  protein kinase A inhibitor peptide, 4  $\mu\text{M}$  R24571, 15  $\mu\text{M}$   $\text{MgCl}_2$ , 100  $\mu\text{M}$  adenosine triphosphate, 0.4  $\mu\text{g}$  myelin basic protein (all from Upstate Biotechnology), and 10  $\mu\text{Ci}$  [ $\gamma$ - $^{32}\text{P}$ ]adenosine triphosphate (3000 Ci per mM, NEN). Reactions were incubated at 30°C with shaking for 20 min, briefly centrifuged, and spotted (30  $\mu\text{l}$ ) in the center of a 2 cm  $\times$  2 cm square of P81 paper (Upstate Biotechnology). Assay squares were washed three times in 0.75% phosphoric acid, once in acetone, and scintillation counted.

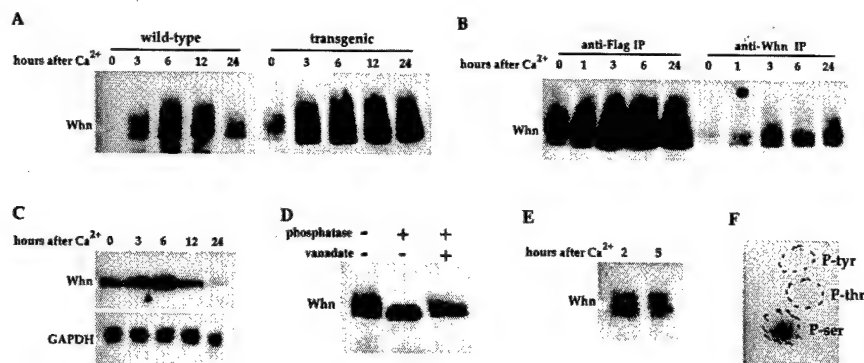
**Northern blot analysis** Total RNA was isolated from keratinocytes using RNeasy mini spin columns (Qiagen, Valencia, CA). Thirty micrograms of total RNA was separated on 1.2% agarose/formaldehyde gels and transferred to Hybond N membranes (Amersham Pharmacia Biotech). Blots were hybridized with a  $^{32}\text{P}$ -labeled *whn* probe corresponding to nucleotides 97–2038 of the mouse cDNA sequence. A  $^{32}\text{P}$ -labeled GAPDH probe (a 1.3 kb hybridized *Pst*I fragment; Tso *et al*, 1985) was used as a loading control.

**$^3\text{H}$ -thymidine labeling** Cells were incubated with 0.5  $\mu\text{Ci}$   $^3\text{H}$ -thymidine (specific activity 5.0 Ci per mmol; Amersham Pharmacia Biotech) for 3 h in 24-well plates. After washing twice with phosphate-buffered saline, proteins were precipitated with 10% trichloroacetic acid and resolubilized in 0.2 M NaOH. Incorporation of  $^3\text{H}$ -thymidine was quantitated by scintillation counting.

**Phosphatase assay** Following immunoprecipitation as described above, Whn protein bound to protein G-sepharose beads was washed in buffer containing 50 mM Tris, pH 8.0, 10 mM  $\text{MgCl}_2$ , 150 mM NaCl, 0.1% Triton X-100, 0.05% SDS, and 2 mM phenylmethylsulfonyl fluoride. Phosphatase reactions were incubated at 37°C for 4 h in buffer containing 50 mM Tris, pH 8.0, 10 mM  $\text{MgCl}_2$ , 150 mM NaCl, and 25 units of calf intestinal alkaline phosphatase (CIAP; Gibco BRL, Gaithersburg, MD). To demonstrate specificity, reactions were performed in the presence or absence of 20 mM  $\text{Na}_3\text{VO}_4$ .

**Phosphoamino acid analysis** Keratinocytes were metabolically labeled with carrier-free  $\text{H}_3^{32}\text{PO}_4$  (1 mCi per ml; Amersham Pharmacia Biotech) in phosphate-free Dulbecco's minimal Eagle's medium (Gibco BRL) containing 1.8 mM  $\text{CaCl}_2$  for 2 h. Whn protein was then immunoprecipitated as described above. Proteins were separated on 7.5% SDS-polyacrylamide gels, transferred to Immobilon membranes, and visualized by autoradiography. Membrane regions containing  $^{32}\text{P}$ -labeled





**Figure 1. *whn* expression is modulated during differentiation *in vitro*.** (A) Analysis of Whn protein. Cell lysates were prepared from wild-type and *inv-whn* cultures before or after the addition of 2 mM calcium (as indicated). Whn was immunoprecipitated using 8 mg (wild-type) or 2 mg (transgenic) of total protein. Immunoprecipitations were electrophoresed on 5% SDS-polyacrylamide gels and immunoblotted with antibodies against Whn. (B) Relative levels of Flag-tagged and endogenous Whn. Transgenic keratinocytes were harvested under low or high calcium conditions as in (A). In each lysate, the Flag-tagged Whn protein was depleted by two sequential immunoprecipitations with Flag M2 monoclonal antibodies (anti-Flag IP). Remaining Whn protein was immunoprecipitated with polyclonal antibodies to Whn (anti-Whn IP). Following electrophoresis on 5% SDS-polyacrylamide gels, the immunoprecipitated proteins were immunoblotted with Whn antibodies. In the anti-Flag time course, each time point represents the combined yield of the two Flag immunoprecipitations. (C) Northern blot analysis of *whn* transcript. RNA was isolated from wild-type keratinocytes before or after calcium treatment. Blots were hybridized with a  $^{32}$ P-labeled murine *whn* cDNA. To normalize the samples, blots were re-probed with a  $^{32}$ P-labeled GAPDH fragment. (D) Whn phosphorylation. Cell lysates were prepared from transgenic keratinocytes after 6 h of calcium treatment. Following immunoprecipitation, Whn was treated with phosphatase in the presence or absence of the phosphatase inhibitor sodium vanadate ( $\text{Na}_3\text{VO}_4$ ). Reactions were electrophoresed on 5% SDS-polyacrylamide gels and immunoblotted using antibodies to Whn. (E) Transgenic keratinocytes were metabolically labeled with  $^{32}\text{PO}_4$  under high calcium conditions. Cell lysates were prepared after the indicated times of calcium treatment, and immunoprecipitations were performed with Whn antibodies. The immunoprecipitated proteins were separated on 7.5% SDS-polyacrylamide gels, transferred to Immobilon membranes, and visualized by autoradiography. (F) Phosphoamino acid analysis of Whn.  $^{32}$ P-labeled residues of Whn were identified by protein hydrolysis and two-dimensional thin-layer electrophoresis. Internal standards for phosphoserine (P-ser), phosphothreonine (P-thr) and phosphotyrosine (P-tyr) are indicated. In wild-type keratinocytes, Whn protein and transcript increase during the early stages of terminal differentiation (A, C). In transgenic cultures, Whn is present at substantial levels under low calcium conditions (A); following calcium treatment, the protein exhibits a strong and prolonged induction, resulting in *whn* overexpression. The Flag-tagged Whn constitutes most of the Whn protein in *inv-whn* cultures (B), demonstrating that the high Whn levels derive directly from the transgene. As shown by phosphatase treatment (D) and  $^{32}\text{PO}_4$  labeling (E), multiple species of Whn are generated by phosphorylation. This phosphorylation takes place primarily on serine residues (F).

Whn were excised, rehydrated in methanol, washed twice with water, hydrolyzed with HCl (110°C, 2 h), and lyophilized (Kamps and Sefton, 1989). Individual  $^{32}$ P-labeled phosphoamino acids were resolved by two-dimensional thin-layer electrophoresis as previously described (Cooper *et al.*, 1984).

## RESULTS

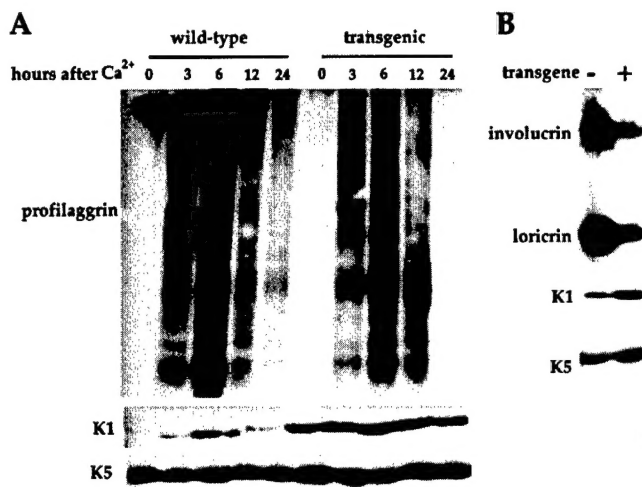
**Induction of Whn during terminal differentiation** To determine the *whn* expression pattern *in vitro*, primary keratinocyte cultures were analyzed for Whn protein and RNA levels. In medium containing low calcium concentrations (50  $\mu\text{M}$ ), mouse keratinocytes exhibit many of the features of basal epidermal cells, including continuous proliferation even after achieving confluence (Hennings *et al.*, 1980). While consisting mainly of proliferative cells, low calcium cultures also generate more differentiated cells that are ultimately shed into the medium (Roop *et al.*, 1987). Upon increasing the calcium content of the medium to 2 mM (Hennings *et al.*, 1980; Hennings and Holbrook, 1983), confluent cultures arrest growth and initiate a program of terminal differentiation similar to that observed in the epidermis.

Whn protein levels were examined using polyclonal antibodies raised against full-length murine Whn. Similar to other regulatory proteins, the absolute amount of Whn is relatively low in wild-type keratinocytes. Thus to measure Whn levels, the protein was immunoprecipitated from concentrated cell extracts, and the immunoprecipitations were then analyzed on immunoblots. Cell extracts were prepared from wild-type keratinocytes under proliferating (low calcium) and differentiating (2 mM calcium) conditions. As shown in Fig 1(A) (left time course), Whn was weakly detected in growing cultures and strongly induced during keratinocyte differentiation. Whn levels increased rapidly following the addition of calcium, peaking at 6 h, and then decreased by 24 h after treatment. At all time points, Whn resolved into multiple,

closely migrating species, indicating post-translational modification of the protein. In an identical experiment using nude primary keratinocytes, no Whn protein was detected, demonstrating the specificity of the antibodies (data not shown). As shown by northern analyses, Whn transcript levels paralleled the protein profile, although the transcript's changes were less dramatic (Fig 1C). Thus, Whn mRNA and protein are induced during the early stages of terminal differentiation in primary keratinocytes. This expression pattern is essentially identical to that observed in the epidermis, suggesting that *whn* is regulated by similar mechanisms *in vitro* and *in vivo*.

To investigate the effects of *whn* overexpression, Whn protein levels were examined in primary keratinocytes derived from *inv-whn* mice. In the epidermis, *inv-whn* expression is confined to suprabasal (postmitotic) keratinocytes (Prowse *et al.*, 1999), consistent with the *inv* expression pattern (Rice and Green, 1979; Walts *et al.*, 1985; de Viragh *et al.*, 1994). In wild-type keratinocyte cultures, involucrin is induced by calcium treatment (Calautti *et al.*, 1995) but is also detected in a subset of attached cells under low calcium conditions (Brissette *et al.*, 1996), indicating the initiation of differentiation. Like the analysis of endogenous *whn* expression, Whn was immunoprecipitated from *inv-whn* cell extracts and visualized on immunoblots. As shown in Fig 1(A, right time course), Whn was easily detected under low calcium conditions and strongly induced by calcium treatment. At each time point, the protein resolved into multiple species, similar, if not identical, to the Whn of wild-type keratinocytes. As transgenic cultures contain relatively high amounts of Whn, the immunoprecipitations were performed with only 2 mg of total protein extract. In contrast, the detection of Whn in wild-type extracts required 8 mg of total protein (Fig 1A, left time course). At all time points, *inv-whn* cultures possessed higher levels of Whn than wild-type cultures. In addition, the time course of *whn* expression differed between the





**Figure 2. Overexpression of *whn* alters the terminal differentiation program.** (A) Wild-type and *inv-whn* keratinocytes were induced to differentiate by the addition of 2 mM calcium for the indicated times. Total cell extracts were normalized for protein content, electrophoresed on 7.5% SDS-polyacrylamide gels, and immunoblotted with antibodies to profilaggrin, K1, or K5. Compared with wild-type cells, transgenic keratinocytes exhibit a stimulation of K1 and an inhibition of profilaggrin. K1 stimulation is observed in both growing and differentiating cultures. (B) Detached cell populations were collected from confluent wild-type and transgenic cultures maintained in low calcium medium. The cells were harvested under low calcium conditions as high calcium prevents keratinocyte detachment. Collected cells were pelleted by centrifugation and then processed as in (A). Immunoblots were probed with antibodies specific for involucrin, loricrin, K1, or K5. In their detached populations, transgenic cultures possess lower levels of loricrin and involucrin than wild-type cultures. Similar to the attached *inv-whn* cells (A), the detached transgenic keratinocytes also display a stimulation of K1.

transgenic and wild-type keratinocytes. By 24 h after calcium addition, Whn declined significantly in wild-type cultures but remained abundant in *inv-whn* cultures. Thus, high Whn levels persisted longer in transgenic cultures, resulting in strong *whn* expression during later stages of the differentiation program.

The Whn protein encoded by the transgene is tagged at the N-terminus with a Flag epitope, enabling the exogenous protein to be distinguished from endogenous Whn (Prowse *et al*, 1999). To examine the relative amounts of tagged and endogenous Whn in *inv-whn* cultures, transgenic cell lysates were depleted of the tagged Whn by two consecutive immunoprecipitations with antibodies to the Flag epitope. The remaining Whn in these lysates was then immunoprecipitated with the Whn antibodies described above. **Figure 1(B)** compares the Flag and Whn immunoprecipitations by immunoblot analysis. As expected, the Flag immunoprecipitations depleted most of the Whn from the lysates, leaving behind low levels of the protein. Thus, transgene expression was directly responsible for the observed increase in Whn.

As shown in **Fig 1(A, B)**, Whn (both endogenous and tagged) resolves as multiple bands during SDS-polyacrylamide gel electrophoresis, suggesting that the protein normally exists in several forms. Since some, if not all, of these forms may result from post-translational modification, we determined whether Whn undergoes phosphorylation. Whn was immunoprecipitated from either transgenic (**Fig 1D**) or wild-type (not shown) keratinocytes, and the protein was incubated with phosphatase. On immunoblots, phosphatase treatment converted the multiple Whn forms to a single, faster-migrating species, strongly suggesting that the different Whn forms are generated by phosphorylation. Inclusion of sodium vanadate ( $\text{Na}_3\text{VO}_4$ ), a phosphatase inhibitor, in the reaction prevented the phosphatase from altering the mobility of the slower

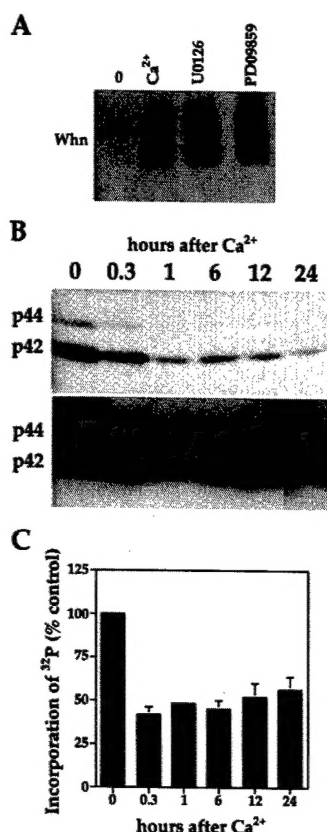
migrating species. To confirm Whn phosphorylation directly, keratinocytes were labeled metabolically with  $^{32}\text{P}$  inorganic phosphate ( $^{32}\text{PO}_4$ ), and the phosphorylation status of Whn was determined by immunoprecipitation and SDS-polyacrylamide gel electrophoresis. As shown in **Fig 1(E)**, Whn was labeled by the  $^{32}\text{PO}_4$ , and the labeled protein displayed a banding pattern similar to that observed on immunoblots. Moreover, the  $^{32}\text{P}$ -labeled Whn generated several phosphorylated fragments when digested with trypsin and resolved by two-dimensional chromatography (phosphopeptide mapping; data not shown). Thus, consistent with its migration on immunoblots, the Whn protein is modified by phosphorylation at multiple sites. To identify the phosphorylated residues, Whn was subjected to phosphoamino acid analysis, and as shown in **Fig 1(F)**, phosphorylation was predominantly on serine residues. On immunoblots, Whn reacted with antibodies to the phosphoryl forms of serine and threonine, indicating that the protein may be phosphorylated on threonine residues as well (data not shown). Thus, phosphorylation produces multiple species of Whn, suggesting complex modulation of this protein's activity. In all, this study represents one of the first biochemical analyses of the Whn protein.

**Effects of Whn on terminal differentiation** As keratinocytes differentiate in the epidermis, the cells migrate towards the surface and proceed through an ordered series of morphologic and biochemical changes. Early terminal differentiation markers, present in deeper layers of the epidermis, include keratins 1 and 10, whereas later markers, associated with the more superficial layers, include filaggrin, loricrin, and involucrin (reviewed in Fuchs, 1990). In culture, wild-type keratinocytes induce both early and late markers in response to calcium (Hennings *et al*, 1980; Hennings and Holbrook, 1983), thus exhibiting a differentiation program similar to the epidermis. To assess Whn's role in terminal differentiation, marker profiles were compared in wild-type and *inv-whn* primary cultures.

Under proliferating (low calcium) conditions, wild-type keratinocytes displayed low levels of K1, and following calcium treatment, this marker increased rapidly, persisting over a 24 h time course (**Fig 2A**). In contrast, transgenic cultures possessed abundant K1 both before and after the induction of differentiation with calcium (**Fig 2A**). At all time points, K1 levels were higher in *inv-whn* keratinocytes than in wild-type cells, and the upregulation of this marker was particularly dramatic under low calcium conditions. Thus, the overexpression of *whn* stimulated an increase in K1, even under conditions that promote proliferation.

Filaggrin, the intermediate filament associated protein, is initially synthesized as a high molecular weight precursor, profilaggrin, which contains many filaggrin repeats (Resing *et al*, 1989). To generate filaggrin, profilaggrin undergoes proteolytic processing and consequently resolves as multiple bands by SDS-polyacrylamide gel electrophoresis. In both wild-type and transgenic keratinocytes, profilaggrin was detected at low levels under proliferating conditions and induced in response to calcium (**Fig 2A**). Nonetheless, a significant difference emerged in profilaggrin levels during differentiation. Compared with wild-type cells, the transgenic keratinocytes displayed a substantial reduction in profilaggrin following calcium treatment. Thus, in contrast to its effect on K1, the *inv-whn* transgene caused a downregulation of profilaggrin in differentiating cells.

During differentiation *in vitro*, wild-type keratinocytes induce involucrin and loricrin (both components of the cornified envelope), but the levels of these proteins are significantly higher in detached cells than in adherent differentiating populations (Roop *et al*, 1987). Thus to examine these markers, detached keratinocytes were harvested from confluent wild-type or *inv-whn* cultures. As shown in **Fig 2B**, transgenic keratinocytes exhibited a dramatic decrease in involucrin and loricrin levels. At the same time, K1 was elevated in detached *inv-whn* cells, consistent with the transgene's effect on adherent differentiating keratinocytes. K5, a marker of the basal epidermal layer, is not modulated in this culture system

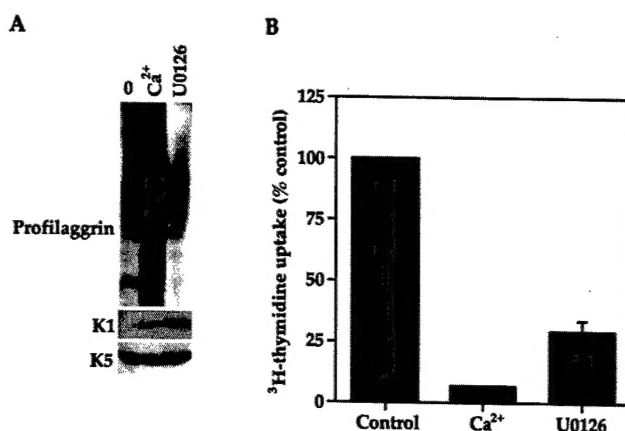


**Figure 3. *whn* expression is modulated by inhibitors of MEK1.** (A) Analysis of Whn protein. Wild-type keratinocytes were treated for 6 h with 2 mM calcium, 1  $\mu\text{M}$  U0126, or 50  $\mu\text{M}$  PD098059. Cell lysates were prepared from treated or untreated cultures, and Whn levels were analyzed by immunoprecipitation/immunoblot as in Fig 1(A). (B) MAP kinase phosphorylation status in primary keratinocytes. Cell lysates were prepared from wild-type cultures at the indicated times after calcium addition (2 mM). Total soluble protein (200  $\mu\text{g}$ ) was electrophoresed on 10% SDS-polyacrylamide gels and immunoblotted using antibodies specific for phosphorylated p42/p44 MAPK. To normalize samples, membranes were reprobed with antibodies that recognize total p42/p44 MAPK (lower panel). (C) MAP kinase activity in primary keratinocytes. Wild-type cultures were treated with 2 mM calcium for the indicated times. p42/p44 MAP kinase was immunoprecipitated from 1 mg total soluble protein and assayed for activity as described in Materials and Methods. The graph expresses the phosphorylation of myelin basic protein as a percentage of the activity found under low calcium conditions, with standard deviation indicated.

(Calautti *et al*, 1995) and served as a loading control in Fig 2(A, B). Thus, like its effect on profilaggrin, *whn* overexpression led to a downregulation of the involucrin and loricrin proteins.

In all, *whn* overexpression resulted in higher levels of an early differentiation marker (K1) and decreased levels of three later markers (profilaggrin, loricrin, and involucrin). These marker profiles indicate that Whn promotes the early stages of differentiation while inhibiting the later stages.

**Effects of the MAP kinase cascade on Whn and terminal differentiation** As shown *in vivo* (Lee *et al*, 1999) and *in vitro* (Fig 1), Whn is induced as epithelial cells make the transition from proliferation to differentiation. Though little is known about the control of keratinocyte fate, the decision to divide or differentiate may be influenced by the p42 and p44 MAPKs. In many cell types, the p42/p44 MAPKs transduce mitogenic signals to substrates such as transcription factors and other kinases (reviewed in Seger and Krebs, 1995; Chang and Karin, 2001). The signals are received from MAP kinase kinases (MEK), which phosphorylate and



**Figure 4. Inhibition of MEK1 causes early features of terminal differentiation.** (A) Wild-type keratinocytes were treated with 2 mM calcium or 1  $\mu\text{M}$  U0126 for 6 h. Total cell protein (50  $\mu\text{g}$ ) was electrophoresed on 7.5% SDS-polyacrylamide gels and immunoblotted with antibodies to K1, profilaggrin, or K5. (B) Wild-type keratinocytes were labeled with  $^3\text{H}$ -thymidine following 24 h of calcium (2 mM) or U0126 (1  $\mu\text{M}$ ) treatment. The graph expresses  $^3\text{H}$ -thymidine incorporation as a percentage, where 100% equals the labeling of untreated cultures.

thereby activate the MAPKs. In human keratinocyte cultures, epidermal growth factor stimulates the activity of p42 MAPK, and this response is blocked by calcium treatment (Medema *et al*, 1994). Moreover, following the introduction of a dominant-negative MEK1, human keratinocytes exhibit reductions in clonal growth and colony-forming efficiency (Zhu *et al*, 1999). These effects correlate with the inhibition of p42/p44 MAPK activity, suggesting that keratinocytes require MAPK signaling to maintain their proliferative potential (Zhu *et al*, 1999). Thus, we investigated whether the p42/p44 MAPK cascade influences *whn* expression.

Wild-type keratinocytes were treated with U0126 (Favata *et al*, 1998) or PD098059 (Alessi *et al*, 1995), which are specific inhibitors of MEK1 and block the activation of its substrates, the p42/p44 MAPKs. Under low calcium conditions, both inhibitors stimulated a great increase in Whn, and these Whn levels were higher than the level observed after calcium treatment (Fig 3A). Thus, the MEK1 inhibitors are the strongest inducers of Whn found to date. As Whn was stimulated by two different inhibitors, it is likely that MEK1 mediates this effect, and that MAPK signaling suppresses Whn levels in proliferating keratinocytes. These results provide the first insight into Whn regulation at the molecular level.

To date, there has been little study of how the MAP kinases affect or respond to keratinocyte decisions. For example, it is not known whether MAPK activity changes as keratinocytes lose proliferative potential or initiate terminal differentiation. Thus to correlate MAPK function with keratinocyte behavior, p42/p44 MAPK status was examined using antibodies specific for their phosphorylated forms. As shown in Fig 3B (upper panel), the p42/p44 MAPKs were phosphorylated under low calcium conditions, indicating that these kinases are active in proliferating keratinocytes. Following calcium addition, the phosphorylated forms of both proteins rapidly decreased and remained low as cells progressed through the differentiation program. This decrease resulted strictly from a change in phosphorylation status, as the total level of MAPK protein was not affected by calcium treatment (Fig 3B, lower panel). To examine MAPK activity directly, total p42/p44 MAPK was immunoprecipitated and assayed for the ability to phosphorylate myelin basic protein. Consistent with the phosphorylation pattern, p42/p44 MAPK activity declined rapidly following the addition of calcium (Fig 3C). Thus, these MAPKs become inactivated as keratinocytes commit to the terminal differentiation program.

As shown in **Fig 2**, *inv-whn* keratinocytes display an altered differentiation program, as early differentiation markers increase and later markers decrease. Since MEK1 inhibitors induce high levels of Whn (analogous to the *inv-whn* transgene), wild-type keratinocytes were examined for their marker profile following U0126 treatment. Under low calcium conditions, the MEK1 inhibitor stimulated an increase in K1 (**Fig 4A**), and this early marker, like Whn (**Fig 3A**), was higher in the presence of U0126 than calcium. Despite this apparent start of differentiation, the U0126-treated cells failed to induce the later marker profilaggrin (**Fig 4A**), instead displaying a small decrease in this protein. In conjunction with this marker profile, U0126 (like calcium) caused a substantial reduction in the incorporation of <sup>3</sup>H-thymidine (**Fig 4B**), showing the arrest of cell division, another early event in the differentiation program. Thus, the inhibition of the MAPK pathway activates early, but not later, features of terminal differentiation, effects essentially identical to the overexpression of *whn*.

## DISCUSSION

During the self-renewal of the epidermis and hair follicles, postmitotic, differentiating cells arise from less differentiated populations capable of proliferation. In this study, we identify a role for Whn in the conversion of progenitor cells to a differentiated phenotype.

In murine epidermis and hair follicles, the induction of *whn* expression correlates with the initiation of terminal differentiation (Lee *et al*, 1999). During epidermal development, *whn* is induced at the same time that K1 first appears (embryonic stage E15.5), and *whn* expression is detected principally in suprabasal cells. As the epidermis matures, *whn* is expressed primarily in the first suprabasal layer, which contains cells in the early stages of terminal differentiation. As epidermal keratinocytes migrate to more superficial layers, *whn* expression disappears, and thus keratinocytes suppress *whn* during later stages of differentiation (Lee *et al*, 1999; Prowse *et al*, 1999).

As shown here, primary keratinocytes also induce *whn* expression during the early stages of terminal differentiation. Under conditions that stimulate proliferation, wild-type cultures possess low levels of Whn, and following calcium treatment, Whn increases dramatically, peaking within 6 h. As cells proceed through the differentiation program, the protein declines, analogous to the *whn* expression pattern in the epidermis. In parallel with the protein, the Whn transcript also rises and falls, but the changes in Whn mRNA appear smaller than the changes in Whn protein. Consistent with the increase in Whn transcript, the Whn promoter (Schorpp *et al*, 1997) contains potential binding sites for AP-1, a transcription factor that activates many genes associated with keratinocyte differentiation (Eckert *et al*, 1997). In all, the results suggest regulation of Whn at several levels. As shown in skin, *whn* expression is controlled, at least in part, at the level of transcription, since Whn promoter activity is induced as cells initiate terminal differentiation (Lee *et al*, 1999). In culture, *whn* may undergo similar transcriptional regulation, since the Whn transcript increases in differentiating cells. Despite this increase, Whn mRNA levels do not correspond precisely to Whn protein levels, as the protein exhibits greater changes than the transcript. Thus, in addition to transcriptional controls, the results suggest the regulation of Whn at the level of translation or protein stability.

In nude epidermis and hair follicles, the differentiating cells exhibit morphologic defects, and some differentiated structures (e.g., the hair cortex and cuticle) often fail to form entirely (Köpf-Maier *et al*, 1990). Consistent with this impaired differentiation, nude skin contains reduced transcript levels of several hair keratin genes (Schlake *et al*, 2000; Schorpp *et al*, 2000). In culture, nude keratinocytes display an abnormal differentiation program, as early markers decrease and later markers increase (Brisette *et al*, 1996). Given these phenotypic effects, we proposed that Whn directly regulates genes associated with terminal differentiation (Lee *et al*,

1999; Prowse *et al*, 1999). Nonetheless, it remained possible that Whn's effects were indirect and that the nude differentiation defects were a secondary consequence of other perturbations. As shown in this study, *whn* overexpression shifts the balance of markers in the differentiation program, suggesting a direct role for Whn in the regulation of differentiation. In *inv-whn* keratinocytes, high Whn levels induce the early marker K1, even under conditions that stimulate proliferation. Concomitantly, the exogenous Whn inhibits profilaggrin, loricrin, and involucrin, three later markers of differentiation. This marker profile is consistent with the epidermal *whn* expression pattern, as Whn is induced during early differentiation stages and repressed during later stages (Lee *et al*, 1999; Prowse *et al*, 1999). Taking these findings together, it is likely that Whn promotes early features of differentiation, directly activating part of the differentiation program. At the same time, Whn appears to suppress or inhibit the later features of differentiating cells. Thus, the results support a role for Whn in the stepwise or temporal regulation of terminal differentiation. That is, Whn can influence a cell's progress through the differentiation program and ensure that events are carried out in the proper sequence.

While we do not know the mechanism by which Whn inhibits later differentiation markers, we note that Whn reduces endogenous involucrin levels without clearly affecting transgene expression, which is driven by the involucrin promoter. The transgene's promoter is carried within a 3.7 kb genomic fragment (Carroll *et al*, 1993), and it is possible that this fragment lacks promoter elements mediating Whn repression. Alternatively, Whn may suppress endogenous involucrin levels through post-transcriptional regulation.

In addition to its induction during terminal differentiation, Whn is phosphorylated at multiple sites, and the phosphorylated residues consist primarily of serine. Whn belongs to the winged-helix/forkhead family of transcription factors (Kaestner *et al*, 2000), and in studies of other family members, phosphorylation modulated transcriptional activity or nuclear translocation (Biggs *et al*, 1999; Brunet *et al*, 1999; Kops *et al*, 1999). As shown by immunoblots, phosphorylation generates multiple species of Whn, which raises the possibility that different species perform different functions.

The differentiation of a cutaneous epithelial cell can be divided into two distinct components: the loss of the ability to multiply and the acquisition of specialized characteristics. In this study, we present evidence that *whn* expression is regulated by a cell's ability to multiply. Whn is strongly induced by two different inhibitors of MEK1, which block activation of the p42/p44 MAPKs. The p42/p44 MAPK pathway is activated through Ras signaling and known to influence cell proliferation as well as other responses to extracellular factors (Chang and Karin, 2001). In human keratinocytes, growth potential decreases following the inhibition of MAPK activity, suggesting a role for MAPK signaling in the maintenance of progenitor populations (Zhu *et al*, 1999). As an extension of this work, we show that the MEK1 inhibitor U0126 arrests the proliferation of murine keratinocytes. Moreover, we demonstrate that MAPK signaling changes dramatically as keratinocytes initiate differentiation. Following calcium treatment, keratinocytes rapidly inactivate the p42 and p44 MAPKs, which implicates these kinases in the switch from a proliferative to a postmitotic state. Taking the results together, the p42/p44 MAPKs perform the related functions of promoting keratinocyte proliferation and suppressing Whn levels. As cells commit to the differentiation program, the MAPK pathway is inactivated, and Whn is induced. This increase in Whn then stimulates the acquisition of specialized characteristics. Thus, MAPK inactivation and Whn induction are part of an early series of steps that convert progenitor cells to a differentiated phenotype.

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